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Identification of Druggable Proteins Regulating Receptor Recycling in Breast Cancer Cells

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14. ABSTRACT

Several recycling regulatory proteins are overexpressed in breast cancer patients and promote the progression of breast cancer. Increased receptor recycling leads to elevated receptors on the plasma membrane, thereby augments signaling cascades important in cancer progression and drives tumor aggressiveness. Inhibition of the altered recycling pathway in breast cancer cells would thus dampen oncogenic signaling and increase the efficacy of therapy. To screen "druggable" proteins regulating receptor recycling, we proposed to monitor EGFR trafficking using a newly developed AP-tag system. We found that while the AP-tag labeling worked for receptor endocytosis studies as described in the original literatures, it could not efficiently label the recycled EGFR. Therefore, this technique is not suitable for studying EGFR recycling as originally proposed.

15. SUBJECT TERMS
Breast cancer, EGFR, receptor recycling, and AP-tag.

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Introduction:

Several recycling regulatory proteins, *e.g.*, Rab11A, Rab25, RCP and Rab11FIP5, are overexpressed in breast cancer patients and promote the progression of breast cancer. Increased receptor recycling leads to elevated receptors on the plasma membrane (PM), thereby augments signaling cascades important in cancer progression and drives tumor aggressiveness. Some ErbB2 targeting drugs inhibit breast cancer partially through shunting the receptor away from recycling into lysosomal degradation. However, the inherent recycling tendency of ErbB2 limits the effectiveness of treating cancer cells with their inhibitors. Inhibition of the altered recycling pathway in breast cancer cells would thus dampen oncogenic signaling and increase the efficacy of therapy. However, Rabs and their binding-proteins are traditionally hard to target. Identification of "druggable" proteins regulating receptor recycling is needed to target this distinct hallmark of breast cancer. A recently developed method, AP-tag (or Avitag), offered specific and covalent attachment of biotin by *E. coli* biotin ligase (BirA) to a surface protein of interest fused with only a 15-amino acid peptide, providing simplicity and extraordinary versatility to study the trafficking of surface proteins in live cells. We proposed to develop an AP-tag system to monitor EGFR trafficking, and planed to screen proteins responsible for dysregulated receptor trafficking in breast cancer cells. The success of the proposal would provide new therapeutic targets from breast cancer.

Body:

The project was unexpectedly delayed in the beginning because we got the incorrect AP-EGFR construct from the investigator who originally reported the AP tag technique. Since this construct was used in several publications, we used it to generate stable cell lines directly. However, after spending 2-3 months on generating stable cell lines, we found out that this construct did not make a full-length EGFR protein. Sequencing the plasmid revealed a point mutation that made a truncated EGFR protein. We spent a lot of time on trouble shooting and re-making new constructs in our lab. Fortunately, the new construct we generated appears to behave as expected.

After generated several versions of constructs with the AP-tag fused to different locations of the EGFR N-terminus, we have successfully identified one of the best constructs. Using BirA purified from bacteria, we could use the AP-tag to follow the endocytosis and recycling of EGFR (Figure 1). One challenge for library screening is heterogeneous responses. Thus stable cell lines derived from a single colony would help to reduce the heterogeneity during siRNA library screening. Therefore, we infected MDA-MB-231 breast cancer cells with lentivirus carrying AP-EGFR, and established 11 stable cell lines with different expression levels of AP-EGFR (Figure 2).

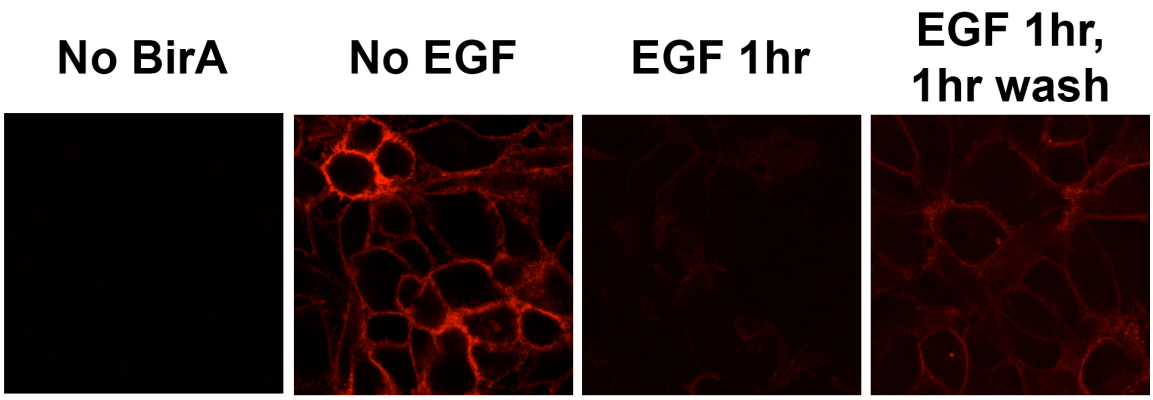

Figure 1. The AP-EGFR on the cell surface can be labeled by biotin and Alexa 594-conjugated streptavidin. MDA-MB-231 cells were infected with lentiviruses expressing AP-EGFR and selected with puromycin. The surface AP-EGFR was visualized by biotin/Alxa 594-strepvatavidin without cell permeabilization. Cells expressing AP-EGFR were visualized in the following conditions: unstimulated, stimulated with EGF for 30 min (internalization), or 1 hr after washing out EGF (pre-treated cells with EGF for 1 hr).

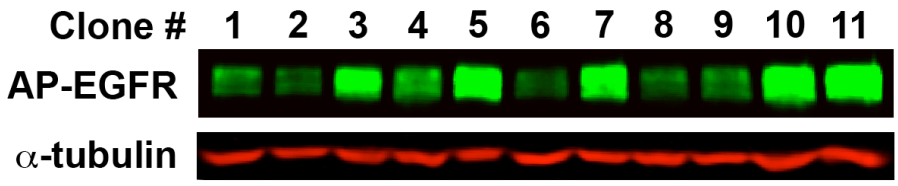

Figure 2. Establishment of MDA-MB-231 cells stably expressing different levels of AP-EGFR. MDA-MB-231 cells expressing AP-EGFR were serially diluted. Stable cell lines expressing different levels of AP-EGFR were established from individual clones. The AP-EGFR was detected with an AP tag antibody followed by the IRDye800 anti-mouse secondary antibody, and visualized using a LI-COR Odyssey imaging system.

We then chose several clones to measure the labeling of EGFR before and after EGF stimulation, and after EGF stimulation and then washout. A problem we kept having was that fluorescent signal from the recycled EGFR was too weak and was only slightly higher than the background. The weak signal actually can only be differed from background with significant noise reduction using the algorithm supplied by the confocal microscope manufacturer. Since the fluorescent signal of the labeled EGFR was much brighter before EGF stimulation, the reason for the weak signal after EGF washout might be caused by the fact that only a small fraction of the internalized EGFR was recycled back to plasma membranes. To enhance the labeling signal of the recycled receptors, we also tried to increase the concentrations of both BirA and biotin and labeling time. However, none of our attempts was able to increase signal to background ratio. We therefore conclude that although the AP-tag labeling works for receptor endocytosis studies as described in the original literatures, it may not be suitable for recycling studies of many receptors that do not efficiently recycle back to plasma membranes after internalization.

Key Research Accomplishments

- Corrected the mutation in the plasmid obtained from the original inventor.
- Tested several EGFR constructs with the AP tag at different locations.
- Set up the labeling protocol in the lab.
- Established stable cell lines expressing AP-EGFR.
- Concluded the limitation of AP-tag labeling in EGFR recycling study.

Reportable Outcomes

The research progress was reported as an abstract and a poster at the 2011 DOD Era of Hope meeting. Several stable cell lines expressing AP-EGFR were created.

Conclusion

We have developed the AP-EGFR labeling strategy to follow the endocytosis with the support of this one-year high-risk concept grant. This system will be used to study the contribution of EGFR endocytosis to breast cancer progression. However, the AP-EGFR strategy is not a good approach for the large-scale study of EGFR recycling as proposed due to its low sensitivity.

References

N/A

Appendices

None

Supporting data

None